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Molecular characterization of virulence genes in *Pseudomonas* species isolated from bulk tank milk in El Menofia governorate.

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ABSTRACT

Pseudomonas is a genus of Gram-negative bacteria belonging to the family Pseudomonadaceae. It is one of the psychrophilic bacteria that can thrive in low-temperature milk in addition to producing heat-resistant spoiling enzymes due to the presence of virulence genes. So, the aim of this study is to characterize the virulence potential of Pseudomonas species isolated from bulk tank milk. Four strains of pseudomonas species were identified as Pseudomonas aeruginosa, Pseudomonas fluorescence, Pseudomonas putida, and Pseudomonas diminuta in the percentages of 16, 12, 4 and 4 (%,) respectively. The pathogenesis of Pseudomonas species is closely related to its virulence genes. In the present study, the virulence genes that were identified in the isolated samples were outer membrane lipoprotein L (oprL) and exotoxin S (exoS) virulence genes which detected in all studied strains, while exo polysaccharide synthesis locus (pslA) virulence gene was detected in 80% of the studied strains, on the other hand exotoxin A (toxA) virulence gene was detected in 70% of the studied strains. Moreover, the elastase B (lasB) virulence gene was detected in 20% of the studied strains.

1. INTRODUCTION

Storage of raw milk under refrigeration conditions is globally practiced for controlling mesophilic and thermophilic bacteria in raw milk, but chilling also provides ideal conditions for the growth of psychrotrophic bacteria. Psychrotrophic bacteria found in raw milk include the gram-negative genera Pseudomonas *(*Ribeiro et al., 2018), The most frequent bacterium that produces heat-resistant spoiling enzymes in cold raw milk is pseudomonas (Machado et al., 2017). Pseudomonas dwells in plants, soil, and is even isolated at a high rate from milk just after milking or after chilling (DoganandBoor, 2003). The presence of *Pseudomonas* species in raw milk is a topic of research on a global scale because of the challenges in controlling their growth during cold storage and the resulting harm to fluid milk (Oliveira et al., 2015). Pseudomonas species have an economic importance through the secretion of protease and lipase enzymes that compromise the integrity of milk ingredients **(**Baur et al., 2015**)**. Until now, *P. aeruginosa* virulence factors have been identified using standard uniplex PCR (Bradbury et al., 2010).

Pseudomonas species contain many virulent genes that add to their pathogenicity. Some of the most common virulence genes found in *Pseudomonas* are*opr*L, *exo*S, *psl*A, *tox*A, and *las*B (Esfahaniet al., 2023)

*Opr*L gene is one of the genes that can be used for PCR identification of clinical *P. aeruginosa*. It is a major constituent of outer membrane lipoproteins of *P. aeruginosa a* (Nikbinet al., 2012). *Las*Bis is involved in proteolysis and elastolysis and is the first gene involved in these processes to be cloned and sequenced. (Toder et al., 1994). It possesses elastolytic action and damages the cell's structural proteins (Benie et al., 2017). *tox*A is an exotoxin that can cause tissue damage and severe infections (Nikbin et al., 2012). *Psl*A is a gene that encodes the production of the extracellular matrix of biofilms (Seyed et al., 2021)

*Exo*S is a type III secretion system effector protein involved in the inhibition of phagocytosis and the induction of apoptosis (Jurado-Irene, 2021). This virulence gene works against host defenses, causing direct harm to host tissues or increasing the bacterium's competitiveness.

Although these bacteria are rendered inactive once milk has been pasteurized and sterilized when raw milk is stored, they secrete a lot of thermo-tolerant lipolytic and proteolytic enzymes, which reduces the shelf life of milk (Wiedmann et al., 2000). The purpose of this work is to study the virulence genes that determine the pathogenicity of Pseudomonas strains isolated from bulk tank milk.

2. MATERIAL AND METHODS

2.1. Collection of samples

From various milk collecting facilities in the El-Menofia governorate, 50 random samples of bulk tank milk were collected ~~at~~ during the winter season (November and December 2021) each sample was kept separate in a plastic bag before being aseptically transferred to the lab in an insulated ice box, where it was allowed to thaw in a refrigerator at (2-5°C). All collected samples were bacteriologically examined for *Pseudomonas* species isolation.

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2.2 Preparation of samples (APHA, 2004)
Ten ml of the sample was put into a sterile jar holding 90 ml of sterile 0.1% peptone water [HI-MEDIA (RM001)]under strictly aseptic conditions. At room temperature (20°C), the mixture was homogenized for 2.5 minutes before being let to stand for 5 minutes.

2.3 Pseudomonas species isolation and identification
A loopful (0.1 ml) from each homogenized sample was streaked onto *Pseudomonas* agar base (Cetrimide agar medium) supplemented with glycerol and incubated at 37°C for 24 hrs. The suspicious colonies were purified and subcultured onto nutrient agar slopes. For further identification, the pure colonies were examined microscopically (APHA, 1992), by motility test (McFadden, 1976). Using semisolid nutrient agar tubes and incubation at 37 °C for 24 hours, microbiologically according to Krieg & Holt (1984). Biochemically (catalase, oxidase, *Triple Sugar Iron* (TSI), Indole and H2S, methyl red (MR), and urease) according to Quinn et al. (2002). The suspected colony was inoculated onto semisolid nutrient agar for preservation.

2.4. Molecular Detection of Virulence Genes.
Five specific primers (Metabion, Germany) of *tox*A, *opr*L, *exo*S, and *psl*A genes were found. Ten *Pseudomonas* isolates (randomly) were examined for detection of virulence genes (*tox*A, *opr*L, *exo*S, and *psl*A)PCRprograme was done using the primers shown in Table (1) and running conditions as detailed in Table (2). Applying 1.5% agarose gel electrophoreses (Sambrook et al. 1989), Emerald Amp GT PCR master-mix (Takara, Japan), and the QIA amp® DNA Mini Kit (Qiagen, Germany, GmbH; Catalogue no. 51304) instructions. Aliquots of amplified PCR products were electrophoresed in 1.5% agarose gel (ABgene) in 1x TBE buffer at room temperature. For gel analysis, 15µl of PCR products were loaded in each gel slot. A100 bp DNA Ladder (QIAGENInc, Valencia, CA, USA) was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

3. RESULTS

3.1. Pseudomonas species identification
Table (3) revealed that all isolated *Pseudomonas species* were catalase, oxidase, and *Triple Sugar Iron* (TSI) positive and negative for Indole and H2S. *Pseudomonas species* were methyl red (MR) negative except *P. fluorescence* (positive) and urease negative except *P. aeruginosa* (positive). Pseudomonas species grew at 4°C except *P.* aeruginosa grew at 42°C. *P. aeruginosa* produced pigment while *P. putida* didn't. *P.diminuta, P.flourescence* and *P. aeruginosa* were Gelatin Liquefying while *P. putida* was not oil adjuvant.

3.2. The incidence of *Pseudomonas* species isolated from bulk tank milk.
In table (4) the incidence of *P. aeruginosa, P. fluorescence, P. putida* and *P. diminuta* were 16%, 12%, 4%, 4%, respectively.

3.3. Molecular characterization of virulence genes of pseudomonas species
In Fig. (1 and 2) the *exo*Sand*opr*L virulence genes were detected in all examined pseudomonas isolates (n: 1-4) *P. aeruginosa,* (n:5-8) *P. fluroscence,(n:9*), *P. putida* and (n:10) *P. diminuta.*
In Fig. (3) *psl*A virulence gene was detected in *p.* aeruginosa, *P.* fluroscence, *P. diminuta* while absent in *P. putida.* Moreover, in Fig (4) *las*B virulence gene was detected in *P. aeruginosa.* Furthermore, in fig. (5). The *tox*A virulence gene was detected in *P.* aeruginosa, *P. fluroscence,* and *P. putida* while absent in *P. diminuta.*
According to Table (5), the *opr*L and *exo*S virulence genes were detected in all ten strains (100%), the *psl*A virulence gene was detected in 8 of the 10 strains (80%), and the *tox*A virulence gene was detected in 7 of the 10 strains (70%). Furthermore, the *las*B virulence gene was found in two of the ten strains studied (20%).

4. DISCUSSION

Pseudomonas spp. is the most prevalent bacteria discovered in cold raw milk that has been demonstrated to produce heat-resistant spoiling enzymes globally (Machado et al. 2017).
Despite the fact that pasteurization and sterilization render these bacteria inactive, they nonetheless produce a large quantity of thermo-stable lipolytic and proteolytic enzymes when fresh milk is kept, which has a detrimental influence on its shelf life or quality. (Wiedmann et al. 2000).
The biochemical profile, such as sugar fermentation or extracellular pigmentation synthesis, was identical to that previously described by Austinand Austin (2007) and DarakandBarde (2015). The presence of pseudomonas sp. Gram-negative bacteria is a sign of either insufficient pasteurization or post-pasteurization contamination in milk products (Doganand Boor, 2003). Pseudomonas-specific extracellular and cell-associated virulence factors increase the pathogenicity and severity of the infection.
The *opr*L and *exo*S virulence genes for Pseudomonas spp. were found by PCR in all strains under study. Similar findings were made by Abdullahi et al. (2013), and Abd El Tawab et al. (2016), while Banerjee et al. (2017) found a lower result (36.8%).
The fundamental resistance of *P. aeruginosa* to antibacterial treatments is due to *opr*L gene as it constitutes the outer membrane lipoprotein which is responsible for the inherent resistance of *P.* aeruginosa to antibiotics of *P. aeruginosa* (Vanderwoude et al. 2020). The *tox*A gene, an inherent genetic sequence located on *P.* aeruginosa chromosome and regulating the synthesis of exotoxin (*exo*S), has been widely used as a target for *P.*aeruginosa detection in PCR methods (Xu et al. 2004). The *exo*S,*tox*A genes were reported in the present study with a percentage of 100% and 70%, respectively. These results were supported by Xu et al. (2004) who stated that the *tox*A gene is produced by the majority of *P.* aeruginosa strains. These results agree with BakheetandTorra (2020) who recorded high distribution of toxA gene among *P.*aeruginosa isolates with rates of 83.3%. A higher prevalence of toxA gene in P. aeruginosa strains (100%) was recorded by Sabharwal et al. (2014).
The results of PCR for amplification of *psl*A of Pseudomonas spp. revealed that *psl*Agene was amplified in 80% of the examined strains nearly results were recorded by Emami et al. (2015). Also, *tox*A gene of *Pseudomonas spp.* revealed that the *tox*A gene was amplified in 70 % of the strains. Similar results were agreed with Gamal et al. (2022) and lower results were recorded by Teymournejad et al. (2016).
Elastase B (Las B) gene was amplified in two out of the ten examined strains. Younis et al. (2015) showed similar results while Heidary et al. (2016) reported that all examined isolates were found to be Las B positive. The LasB gene

encodes the zinc metalloprotease Las Belastase, which targets eukaryotic proteins and degrades cell structural proteins. On lung tissue, LasB elastase displays elastolytic activity (Leduc et al. 2007).

5. CONCLUSIONS

Pseudomonas species mostly produce proteolytic enzymes that will have an impact on the quality of the product and public health hazards for the consumer. So, milk should be properly pasteurized and proper hygienic measures, such as the implementation of HACCP, and applying GMPs during the dairy products' manufacture. Applying ISO 22000 in dairy shops will prevent spoilage of food and extend the dairy products' shelf-life. Personal hygiene education for food handlers is critical for food safety.

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